Food Chemistry 114 (2009) 516–522

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Identification of pro-drug type ACE inhibitory peptide sourced from porcine myosin B: Evaluation of its antihypertensive effects *in vivo*

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ARTICLE INFO

Article history:
Received 24 June 2008
Received in revised form 1 August 2008
Accepted 25 September 2008

Keywords:
Myosin B
Myosin
Actin
Antihypertensive activity
Pro-drug peptide
ACE inhibitory activity

ABSTRACT

This study aimed to identify a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from porcine skeletal myosin B. Proteins were hydrolyzed with pepsin and the hydrolysates were then subjected to various types of chromatography to isolate the active peptides. The 50% inhibitory concentrations of Lys–Arg–Val–Ile–Gln–Try (M6 a novel peptide) and Val–Lys–Ala–Gly–Phe (A5, a peptide discovered by Ukeda et al. (1991)) were 6.1 and 20.3 μM, respectively. As a result of a homology search, it was determined that the M6 peptide originated from myosin and peptide A5 was of actin origin. M6 is a novel ACE inhibitory peptide, whose activity was shown to be the strongest amongst the previously published myosin-originated peptides. Kinetic evaluations showed that both peptides are competitive inhibitors of ACE. Based on their activity against ACE, M6 was classified as a pro-drug conformer and A5 was classified as a substrate conformer. When both peptides were administered orally to spontaneously hypertensive rats at doses of 10 mg/kg, temporal hypertension was observed after 6 h. This study suggests that M6 and A5 are peptides that may serve several purposes. Based on their remarkable antihypertensive activity, we suggest that M6 and A5 may have potential applications as functional food, which could be used as nutraceutical compounds.

1. Introduction

Recently certain indexes were found to indicate that 25% of the Japanese population suffers from high blood pressure (Muguruma, Ahhmed, & Kawahara, 2008). Cardiovascular diseases represent the first cause of morbidity and mortality in Western countries, with hypertension affecting about 20% of the world adult population (Miguel, Alonso, Salaices, Aleixandre, & Lopez-Fandin, 2007). It is essential to reduce the incidence of high blood pressure among the population as this condition can lead to damaging effects on the brain, heart and blood vessels. Because of these effects, high blood pressure is a major cause of human mortality especially among the elderly. More accurately, this disease induces cerebrovascular incidents, heart failure and kidney disease, which could all lead to more complicated dysfunctions of the internal organs. High blood pressure diseases can be classified into two main groups, the first group which makes up approx. 10% of total cases, can be described as the essential group in which the causes of high blood pressure have been identified, and the second section which makes up 90% of total cases, can be regarded as the secondary intricate group in which the causes of the condition have not yet been identified (Kostis, 1995). However, some scientists have been trying to evaluate the real cause that expectedly could be due to life style-related diseases. Angiotensin I-converting enzyme (ACE), which is a dipeptidylcarboxypeptidase, plays an important physiological role in regulating blood pressure (Skeggs, Khan, & Shumaay, 1965). *In vivo*, the increase and decrease in blood pressure conditions is obviously dependent on some crucial metabolites such as angiotensin I-converting enzyme (ACE). The greater the ACE activity, the more angiotensin I is converted to angiotensin II, which induces high blood pressure. ACE converts an inactive form of the decapeptide, angiotensin I, to a potent vasoconstrictor, the octapeptide angiotensin II, and also inactivates bradykinin which reduces blood pressure. For these reasons, specific inhibitors of ACE are useful for regulating physiological activities associated with ACE in the human body (Ondetti, Rubin, & Cushman, 1977).

As a large number of individuals suffer from such disease, scientists believe that other methods rather than chemical and pharmacological medication should be identified in the effort to reduce hypertensive diseases. Therefore, potential biological, functional and nutraceutical methods should be utilized as a treatment to minimize the number of individuals who fall into the hypertensive category and have been afflicted with diseases arising from this

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doi:10.1016/j.foodchem.2008.09.081

condition. In the last 10 years, many researchers worldwide have paid considerable attention to the use of certain food constituents including meats to prevent the action of ACE in elevating blood pressure. Some physiological functions of food constituents have been elucidated, and it is important to use such food constituents for the maintenance and promotion of health. For instance, some peptides have recently been reported to play an important role in controlling the development of hypertension by regulating the rennin-angiotensin system (Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001; Asihda, Yoshimi, Kawano, Matsuoka, & Omae, 1996; Haws, Shaul, Arant, Atiyeh, & Seikaly, 1994; Katayama et al., 2004; Katayama et al., 2007; Katayama et al., 2008). There are of course other studies concerned with ACE inhibitory peptides with a view to enhance the value of food products. Many of these peptides have been sourced from animal products such as bovine casein, porcine muscle, egg white, yak milk casein, sheep milk yoghurt and oyster (Miguel, Contreras, Recio, & Aleixandre, 2009; Miguel and Fidel, 2007; Miguel et al., 2007; Maoa et al., 2007; Papadimitriou et al., 2007 and Wang et al., 2008, respectively).

Controlling blood pressure in this way is useful and beneficial. Katayama et al. (2003) suggested that ACE inhibitory peptides were generated not only from proteins such as myosin and actin but also from regulatory proteins such as tropomyosin and troponin. Although an ACE inhibitory peptide, Arg–Met–Leu–Gly–Gln–Thr–Pro–Thr–Lys (RMLGQTPTK), has been isolated from troponin (Katayama et al., 2003), the hydrolysate of troponin was expected to include other peptides with ACE inhibitory activity because of its variable chromatographic results.

Since this disease is the most common serious chronic health problem as it carries a high risk of cardiovascular complication (Manger & Page, 1982), and as many studies have indicated that certain foods possess the potential to reduce such diseases, we undertook this study to identify and evaluate a peptide which may play a crucial role as an antihypertensive peptide. The objective of this study was to isolate and identify a novel ACE inhibitory peptide from the hydrolysate of porcine myosin B. This peptide was administered to spontaneously hypertensive rats (SHR) to determine whether it worked as an antihypertensive substance *in vivo*.

2. Materials and methods

2.1. Preparations of myosin B

Pork loin muscle (*Longissimus dorsi)* was purchased from Marudai Shimane Farm Co., Ltd. (Shimane, Japan). Myosin B was prepared using a method similar to that described by Ahhmed, Kawahara, and Muguruma (2008). The muscles were minced in a meat grinder (MK-GL 20-W National), and the minced meat was extracted with 3 volumes of cold Weber–Edsall solution (0.6 M KCl, 0.04 M $NaHCO_3$, 0.01 M Na_2CO_3) for 24 h at 4 °C in a refrigerator using a laboratory stirrer (Chemy Stirrer. B-100, Rikakikai Co., Ltd., Tokyo, Japan) at setting 2. Two portions of 0.6 M KCl were then added to the solutions. The mixture was then stirred and then centrifuged at 16,000 rpm for 60 min at 4 °C (Himac CR 20E centrifuge, Hitachi, Tokyo, Japan). The supernatants, which contained myosin B proteins, were collected, and passed through two layers of gauze. The filtrate was subsequently precipitated by dilution with cold water (×10) to give a final ionic strength of ~0.04. The mixture was centrifuged at 5000 rpm for 20 min at 4 °C. The precipitate was collected and dissolved in an appropriate volume of 0.6 M NaCl. Afterwards the mixture was diluted again with cold water to give a final ionic strength ~0.04, and it was similarly centrifuged once more. The precipitate was collected and dialyzed against phosphate buffer saline (PBS; 0.8% NaCl, 10 mM Na-phosphate, pH 7.5) before hydrolysis.

2.2. Hydrolysis of myosin B

Pepsin from porcine gastric mucosa was obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Myosin B from Japanese domestic pork (5 mg/ml) was suspended in PBS, and denatured by heating for 10 min at 98 °C; the pH was adjusted to 2 with 1 M HCl, and pepsin was added in a ratio 1:100 of enzyme to substrate. After 6 h of digestion at 37 °C, the pH of the mixture was again adjusted to 7.5 with 1 M NaOH. Enzymatic activity was terminated by heating for 10 min at 98 °C. The reaction mixture was centrifuged for 20 min at 18,000*g*, and the supernatant was then collected for the ACE inhibitory experiment.

2.3. ACE inhibitory activity assay

The ACE inhibitory activity was measured according to the method of Cushman and Cheung (1971), with slight modifications as described in a previous publication (Katayama et al., 2004). This assay is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (Hip–His–Leu) catalyzed by ACE.

A sample solution of peptide (6 μl) was mixed with 50 μl of 7.6 mM Hip–His–Leu (Nacalai Tesque Inc., Kyoto, Japan) as substrate containing 100 mM sodium borate buffer (pH 8.3) and 608 mM NaCl and then pre-incubated at 37 °C for 5 min. The reaction was initiated by the addition 20 μl of 60 milli-unit/ml rabbit lung ACE (Sigma–Aldrich, Co., MO. USA) in a buffer containing 0.25 M sodium borate buffer (pH 8.3) and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 554 μl 0.1 N HCl to the samples except for the blank (554 μl 0.1 N HCl were added before the pre-incubation). The hippuric acid liberated by ACE was extracted by adding 1.5 ml ethyl acetate to the mixture with vigorous shaking for 2 min. After centrifugation at 3000 rpm for 20 min, 1 ml of the ethyl acetate layer was collected; it was then dried at 100 °C for 10 min. The hippuric acid was dissolved with 1 ml 1 M NaCl and its absorption at 228 nm was determined in a spectrophotometer. The concentration of ACE inhibitors required to inhibit 50% of ACE activity was defined as the IC_{50} value. ACE inhibitory activity was calculated as follows

$$\text{Inhibition}(\%) = \frac{(C-S)}{(C-S)} \times 100$$

S: Absorbance of sample.
C: Absorbance of control (buffer for samples).
B: Absorbance of blank (hydrochloric acid was added before ACE).

2.4. Purification of ACE inhibitory peptide from myosin B hydrolysate

The myosin B hydrolysate was separated by gel permeation chromatography. The peptic hydrolysate of porcine myosin B was applied to a Superdex^{TM} 30 prep grade (GE Healthcare Biosciences, AB, Uppsala, Sweden) column (1.6 × 90 cm) and eluted with a solution of 20 mM sodium acetate (pH 7.0), 150 mM NaCl and 0.5 mM NaN_3 at a flow rate of 0.45 ml/min. Eluted fractions were collected and desalted with 50% CH_3CN using a SEP-PAK Plus C_{18} cartridge (Waters Co., Milford, MA, USA). Aprotinin (6500 Da), oxtocin (1007 Da) and riboflavin (376 Da) were used as molecular weight markers. Next, the active fractions (Nos. 55 and 56) were subjected to reverse phase (RP) high-performance liquid chromatography (HPLC) using a Inertsil ODS-2 (4.6 × 250 mm) column, and were fractioned using a 1–80% gradient of acetonitrile in 0.1% TFA at a flow rate of 0.5 ml/min. Fractions were collected every minute. The active fractions (Nos. 55 and 56) were further separated by RP-HPLC using a 1–50% gradient of acetonitrile in 0.1% TFA at a flow rate of 0.5 ml/min and the same column. The active fractions of both peptides were then subjected to further